THYROID
Volume 24, Number 6, 2014
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DOI: 10.1089/thy.2013.0332

# Luteal Expression of Thyroid Hormone Receptors During Gestation and Postpartum in the Rat

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***Background:*** Progesterone ($P_4$) is the main steroid secreted by the corpora lutea (CL) and is required for successful implantation and maintenance of pregnancy. Although adequate circulating levels of thyroid hormone (TH) are needed to support formation and maintenance of CL during pregnancy, TH signaling had not been described in this gland. We determined luteal thyroid hormone receptor isoforms (TR) expression and regulation throughout pregnancy and under the influence of thyroid status, and *in vitro* effects of triiodothyronine ($T_3$) exposure on luteal $P_4$ synthesis.
***Methods:*** Euthyroid female Wistar rats were sacrificed by decapitation on gestational day (G) 5, G10, G15, G19, or G21 of pregnancy or on day 2 postpartum (L2). Hyperthyroidism and hypothyroidism were induced in female Wistar rats by daily administration of thyroxine ($T_4$; 0.25 mg/kg subcutaneously) or 6-propyl-2-thiouracil (PTU; 0.1 g/L in drinking water), respectively. Luteal TR expression of mRNA was determined using real-time reverse-transcription quantitative polymerase chain reaction, and of protein using Western blot and immunohistochemistry. Primary cultures of luteal cells and of luteinized granulosa cells were used to study *in vitro* effects of $T_3$ on $P_4$ synthesis. In addition, the effect of $T_3$ on $P_4$ synthesis under basal conditions and under stimulation with luteinizing hormone (LH), prolactin (PRL), and prostaglandin $E_2$ ($PGE_2$) was evaluated.
***Results:*** TR$\alpha$1, TR$\alpha$2, and TR$\beta$1 mRNA were present in CL, increasing during the first half and decreasing during the second half of pregnancy. At the protein level, TR$\beta$1 was abundantly expressed during gestation reaching a peak at G19 and decreasing afterwards. TR$\alpha$1 was barely expressed during early gestation, peaked at G19, and diminished thereafter. Expression of TR$\beta$1 and TR$\alpha$1 at the protein and mRNA level were not influenced by thyroid status. $T_3$ neither modified $P_4$ secretion from CL of pregnancy nor its synthesis in luteinized granulosa cells in culture.
***Conclusions:*** This study confirms for the first time the presence of TR isoforms in the CL during pregnancy and postpartum, identifying this gland as a TH target during gestation. TR expression is modulated in this tissue in accordance with the regulation of $P_4$ metabolism, and the abrupt peripartum changes suggest a role of TH during luteolysis. However, TH actions on the CL do not seem to be related to a direct regulation of $P_4$ synthesis.

## Introduction

The corpus luteum (CL) is a transient endocrine gland that plays a central role in the maintenance of pregnancy (1). Progesterone ($P_4$) is the main steroid secreted by this gland and is required for the successful implantation and maintenance of pregnancy (2). In humans, the CL sustains its capacity to synthesize $P_4$ almost throughout the length of pregnancy, nonetheless, its presence is essential only until the eighth week of gestation; luteal failure before this time may result in spontaneous abortion (3). Therefore, disorders related to inadequate $P_4$ secretion by the CL are likely to affect pregnancy outcomes.

Several endocrinologic abnormalities such as thyroid disease have been implicated as etiologic factors of recurrent pregnancy loss (3–9). Hypothyroidism has a statistically significant relationship with recurrent pregnancy loss in the first trimester in humans (10). Hypothyroidism also interferes with the formation and function of CL, resulting in pregnancy failure. Experimental findings indicate that the presence of an

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adequate circulating level of thyroid hormone (TH) is needed to support CL formation and pregnancy (5,6,11).

Previous results from our laboratory demonstrated that hypothyroidism impairs CL function during gestation delaying the onset of parturition in the rat (12,13). On the contrary, hyperthyroidism advances $P_4$ withdrawal and the onset of parturition (14,15). The effects of hyperthyroidism or hypothyroidism on luteal function are mediated by changes in prostaglandin balance. At the luteal level, hyperthyroidism decreased prostaglandin $E_2$ ($PGE_2$) and increased prostaglandin $PGF_{2a}$ ($PGF_{2a}$), while hypothyroidism increased $PGE_2$ and diminished $PGF_{2a}$ at the end of gestation. Collectively, these alterations result in changes in the moment of induction of 20α-hydroxysteroid dehydrogenase (20α-HSD) expression, the enzyme that metabolizes $P_4$ to an inactive metabolite. Thus, in hyperthyroid rats the expression of this enzyme is advanced and in hypothyroid rats it is delayed, resulting in advanced and delayed parturition, respectively (13,14). These data may indicate that THs participate in the maintenance and function of the CL of gestation in rodents.

THs play critical roles in differentiation, growth, and metabolism. The classic genomic actions of THs are mediated by two main nuclear receptor isoforms, TRα and TRβ, that act mainly as hormone-inducible transcription factors. The isoforms of TRα and TRβ are encoded by the *THRA* and *THRB* genes. The TRα1, TRα2, and TRβ1 isoforms are widely expressed whereas TRβ2 is predominantly restricted to the hypothalamus and pituitary. TRα1, TRα2, and TRβ1 mRNA and protein are present in ovarian surface epithelial cells in humans (16), whereas human oocyte, granulosa, cumulus, and stromal cells express TRα1, TRα2, TRβ1, and TRβ2 (17–19). Binding studies showed that triiodothyronine ($T_3$) binds to CL nuclei in a hormone-specific manner indicating a direct effect of THs on human CL (20). Another investigation demonstrated that $T_3$ stimulates $P_4$ release from human luteal cells in an indirect manner, mediated by a putative proteinaceous factor (21). However, the precise role of TR signaling in the CL of gestation remains elusive.

Although the CL of pregnancy is not an established TH target, the evidence reported indicates that TH signaling plays a role on luteal function during pregnancy, thus becoming an issue for further investigation. Therefore, we hypothesized that THs signal in the CL through their known receptors in a canonical manner allowing the normal function of the CL. In this sense, we set out to determine: (1) which TR isoforms are expressed in CL; (2) whether this family of proteins is differentially expressed throughout pregnancy; (3) whether there is differential expression of the TRs at the mRNA or protein level; (4) and if they are regulated by thyroid status. Finally, because $P_4$ is the main product of CL, we also sought to determine whether exposure of $T_3$ might affect luteal $P_4$ synthesis *in vitro*.

## Materials and Methods

### *Animals and experimental design*

Adult female Wistar rats bred in our laboratory, 3–4 months old, weighing 200–230 g at the onset of treatment and with regular 4-day estrus cycles were used. The estrus cycle was monitored by cytologic examination of the vaginal smears obtained by daily vaginal lavage during at least three consecutive estrus cycles before starting the treatments. They were kept in a light- (lights on 6:00 AM to 10:00 PM) and temperature- (22–24°C) controlled room. Rat chow (Cargill, Córdoba, Argentina) and tap water were available *ad libitum*. The presence of spermatozoa in the vaginal smears the morning after caging with a fertile male in the night of proestrus was indicative of pregnancy and this day was counted as gestational day (G) 0 of pregnancy.

To determine the pattern of hormonal secretion and TR expression in the CL during pregnancy, groups of 6–8 euthyroid rats were sacrificed by decapitation within 5 seconds after removal from the home cage on G5, G10, G15, G19, or G21 or on day 2 (L2) postpartum between 10:00 AM and 12:00 noon. In other groups of rats, hyperthyroidism was induced by daily administration of thyroxine ($T_4$; 0.25 mg/kg subcutaneously in saline) and hypothyroidism by administration of 6-propyl-2-thiouracil (PTU) at a concentration of 0.1 g/L in the drinking water; both treatments were started on the estrus day, 8 days previous to mating, and the rats were sacrificed on G19 as described above. Trunk blood was collected and serum was separated by centrifugation and stored at −20°C until used. The CLs were rapidly removed, rinsed in cold saline solution, snap-frozen in liquid nitrogen, and stored at −70°C until they were used for RNA and protein isolation. For cell culture experiments, the fresh CLs were subsequently processed for enzymatic dispersion. For immunohistochemistry analysis, one entire ovary per rat was removed from other groups of G19 control, hypothyroid, and hyperthyroid animals ($n = 3$) and immediately fixed in 10% buffered formalin at room temperature.

Animal maintenance and handling was performed according to the Guiding Principles in the Care and Use of Laboratory Animals of the U.S. National Institutes of Health (NIH publication no. 86-23, revised 1985 and 1991) and the United Kingdom's requirements for ethics of animal experimentation (Animals Scientific Procedures, Act 1986). All procedures were approved by the Institutional Animal Care and Use Committee of the School of Medical Science, National University of Cuyo, Mendoza, Argentina (Protocol approval no. 17/2012).

### *Hormone determinations*

Prolactin (PRL) and thyrotropin (TSH) were measured by double-antibody radioimmunoassay (RIA) using materials generously provided by Dr. Parlow and the National Hormone and Pituitary Program, Harbor-UCLA Medical Center, Torrance, CA, as previously described (12).

$P_4$, $T_3$, and $T_4$ concentrations in sera were measured by radioimmunoassay using commercial kits for total hormones (DSL-3400, DSL-3100, and DSL-3200 double-antibody radioimmunoassay, respectively, from Diagnostic System Laboratories (Webster, TX) as previously described (11).

### *RNA isolation and CL analysis*

Reverse-transcription quantitative polymerase chain reaction (RT-qPCR) analysis was used to detect the presence of TR isoforms in CL of pregnancy. Total RNA was extracted from 100 mg of deep-frozen CL, using TRIzol® reagent (Life Technologies, Carlsbad, CA), and following the instructions provided by the manufacturer. The integrity of the total RNA was determined by electrophoresis on 2% (w/v) agarose gels stained with ethidium bromide. Nucleic acid concentrations

were measured at 260 nm. Purity of the total RNA extracted was determined as the 260:280 nm ratio with expected values between 1.8 and 2.

Five micrograms of total RNA were reverse-transcribed to cDNA in a 25 $\mu$L volume reaction in the PTC-100 Programmable Thermal Controller (MJ Research Inc., Bruno, Canada). The RNA was first denatured at 70°C for 5 minutes in the presence of 2.5 $\mu$g of random hexamer primers (Invitrogen Life Technologies, Carlsbad, CA). For the subsequent RT reaction the following mixture was added: RT buffer (50 mM Tris-HCl [pH 8.4], 75 mM KCl, 3 mM $MgCl_2$), 0.5 mM dNTPs, 5 mM DTT, 200 units M-MLV Reverse Transcriptase (Invitrogen Life Technologies). The reaction was incubated at 37°C for 50 minutes and then inactivated by heating at 70°C during 15 minutes. The cDNA was stored at −20°C. PCRs were performed using a Corbett Rotor Gene 6000 Real-Time Thermocycler (Corbett Research Pty Ltd. Sydney, Australia) using Eva-Green (Biotium, Inc., Hayward, CA) in a final volume of 20 $\mu$L. The reaction mixture consisted of 2 $\mu$L of 10× PCR buffer, 1 $\mu$L of 50 mM MgCl2, 0.4 $\mu$L of 10 mM dNTP Mix (Invitrogen Life Technologies), 1 $\mu$L of 20× Eva Green (Biotium, Inc., Hayward, CA), 0.25 $\mu$L of 5 U/$\mu$L Taq DNA Polymerase (Invitrogen Life Technologies), 0.1 $\mu$L of each 2.5 mM primer (forward and reverse primers) and 10 $\mu$L of diluted cDNA. The PCR reactions were initiated with 5-minute incubation at 95°C, followed by 40 cycles of 95°C for 30 seconds, 60°C for 30 seconds, and 72°C for 30 seconds. A melting curve was performed at the end of the PCR run over the range of 55–95°C, increasing the temperature stepwise by 0.5°C every 2 seconds. Gene-specific amplification was confirmed by a single peak in the melting-curve analysis and a single band on a 2% agarose gel stained with ethidium bromide. All pairs of primers listed in Supplementary Table S1 (Supplementary Data are available online at www.liebertpub.com/thy) had been previously published by other authors as follows: *TR$\alpha$1, TR$\alpha$2* and *TR$\beta$1* (22); *glyceraldehyde-3-phosphate dehydrogenase (Gapdh)* (23); *ribosomal protein L19 (Rpl19)* (24), *hypoxanthine phosphoribosyl transferase 1 (Hprt1)* (25), and *ribosomal protein S16 (Rps16)* (14).

All qPCR reactions were performed in duplicate and Cq values were averaged; every run also included a nontemplate control and a control for cDNA contamination that contained a pool of RNA of the samples and was subjected to the protocol of retro-transcription exchanging the retrotranscriptase for water in the reaction mixture. All qPCR assays were validated *in silico* using the RTPrimerDB primer and BLAST evaluation, checking primer specificity. The data acquisition of the expression levels was done employing the second derivative maximum method as computed by the Rotor Gene 6000 Rotary Analyzer Software version 1.7.87 (Corbett Research Pty Ltd., Sydney, Australia). For subsequent data analysis, the values provided by the instrument were imported into a Microsoft Excel spreadsheet and analyzed using the $2^{-\Delta\Delta Cq}$ method (26). Fold change in normalized mRNA expression of the different genes of interest, relative to the expression at early gestation (G5), was calculated for each sample throughout gestation, and relative to the control group when assessing the difference among thyroid status.

A dilution series was created from a pool of cDNA made from aliquots of all of our samples in order to construct standard curves for each primer pair. In brief, qPCR reactions were performed in duplicate for each serial dilution and were plotted against the logarithm of the cDNA dilution factor. The amplification efficiency for each gene evaluated was calculated from the expression $[10(1/-S) - 1]$, in which $S$ represents the slope of the linear regression obtained.

The expression stability of the four candidate reference genes, *Gapdh*, *Rpl19*, *Hprt1,* and *Rps16* were estimated using the freely available online software BestKeeper version 1 (http://gene-quantification.com/bestkeeper.html). This approach allowed us to select *Gapdh* as the reference gene since it showed low Cq variation along gestation and between treatment groups on G19 compared to *Rps16*, *Hprt1*, and *Rpl19* (Supplementary File S1).

### *Western blot analysis of TH receptors*

TR$\alpha$1 and TR$\beta$1, the predominant TH binding receptors, were analyzed by Western blot at the luteal level in order to determined their protein expression and regulation during pregnancy. TR proteins were expressed as the ratio of signal intensity for the protein relative to that of $\alpha$-tubulin. Protein samples used for the detection of TRs during gestation were isolated from the phenol-ethanol supernatant obtained from RNA isolation using TRIzol reagent following the manufacturer's instructions.

Protein samples used for the detection of TR on G19 in hypothyroid, hyperthyroid, and control rats were isolated from CL homogenized in 10 volumes of lysis buffer that contains 50 mM Tris-HCl (pH 7.5), 150 mM NaCl, 0.5% IGEPAL, 50 mM sodium fluoride, and a protease inhibitor cocktail (Sigma P8340, Sigma-Aldrich, St. Louis, MO). The lysates were incubated on ice for 30 minutes and then another 30 minutes on ice on a rocking platform. Lysates were centrifuged at 10,000 rpm for 15 minutes at 4°C, and the supernatant was considered whole cell extract. Protein content was assessed by the bicinchoninic acid method (BCA; Pierce, Rockford, IL). The whole cell extract was aliquoted and stored at −80°C. Before loading, the proteins were boiled for 10 minutes in sample buffer and an amount of 50 $\mu$g of proteins were separated in a 12% (w/v) acrylamide gel by sodium dodecyl-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to polyvinylidene difluoride (PVDF) membranes. The blots were blocked in 5% (w/v) nonfat milk in tris-buffered saline (TBS) containing 0.1% (v/v) Tween-20. Blots were probed overnight with the appropriate dilution in 2.5% bovine serum albumin (BSA) of each of the primary antibodies. The membranes were washed 3×5 minutes in TBS-T and incubated with 1:5000 dilution of a peroxidase conjugate secondary antibody (anti-mouse horseradish peroxidase [HRP] from Cell Signaling [Beverly, MA] and anti-rabbit HRP from Santa Cruz Biotechnology [Dallas, TX]) for 1 hour at room temperature. The blots were washed, developed by chemiluminiscence, using a ChemiDoc XRS+ System (Bio-Rad Laboratories, Hercules, CA). Densitometric analysis of the protein bands was performed using Image Lab Software version 4.0 from Bio-Rad Laboratories expressing the results as the ratio of signal intensity for the different TR proteins relative to that of $\alpha$-tubulin. The following dilutions were used for the primary antibodies: TR$\alpha/\beta$ (FL-408; 1/1000), TR$\beta$1 (J52; 1/1000) from Santa Cruz; TR$\alpha$1 (PA1-211A; 1/1000) from Thermo Scientific (Waltham, MA), and $\alpha$-tubulin (T6040; 1/12000) from Sigma-Aldrich.

### *Immunohistochemistry*

To determine the cellular localization of the TRs, we performed immunohistochemistry (IHC) analysis in paraffin sections of whole ovaries. The tissues were fixed in 10% buffered formalin at room temperature, and processed for paraffin embedding. Five-micrometer sections were cut in a Reichert-Jung Hn 40 microtome and mounted onto 3-aminopropyltriethoxysilane-coated slides (Sigma-Aldrich) for subsequent IHC analysis using the mouse monoclonal antibody TR$\beta$1 (J52).We performed IHC in these samples using the antibody sc-772 that recognizes both TR$\alpha$1 and TR$\beta$1 isoforms, and PA1-211A that recognizes specifically TR$\alpha$1, but we did not observe a reliable signal with any of these antibodies (data not shown).

TR antigen unmasking was carried out in 0.01 M citrate buffer (pH 6.0) at 100°C for 25 minutes. The slides were incubated with the primary antibody overnight at 4°C in a humidity chamber at 1/200 dilution. Nonspecific mouse immunoglobulin G1 (IgG1) antibody and purified rabbit preimmune serum (Dako, Kingsgrove, NSW, Australia) were used as isotype controls. Secondary biotin-conjugated goat anti-mouse/rabbit antibodies (Vector Laboratories Homebush, NSW, Australia) were used at 1/250 dilution. Specific primary-secondary antibody complexes were detected using ABC reagent (Vector) and visualized using a DAB peroxidase substrate kit (Vector). Slides were lightly counterstained with hematoxylin to reveal nuclei and observed with a Nikon Eclipse E200 microscope (Nikon Instruments Inc., Melville, NY) and processed with Micrometrics SE Premium Software (Accu-Scope Inc., Commack, NY).

### *Primary luteinized granulosa cells and luteal cell culture*

To determine a possible direct effect of THs on luteal cell $P_4$ synthesis, primary luteinized granulosa cells were cultured as previously described (27). Briefly, 25-day-old immature rats were treated with 10 IU pregnant mare serum gonadotropin (PMSG) intraperitoneally followed by 10 IU human chorionic gonadotropin (hCG) intraperitoneally 2 days later. Eight hours after hCG injection, follicles were gently extruded from the ovaries under a dissecting microscope, incubated sequentially in DMEM/F12 (1:1) containing, respectively, 6 mM ethylenediaminetetraacetic acid (EDTA) and then in 0.5 M sucrose, and luteinized granulosa cells were harvested by needle-pricking the follicles. The cells were cultured for 48 hours at 37°C in an atmosphere of 5% $CO_2$/95% air in DMEM/ F12 (1:1) with 15 mM HEPES, 1% fetal bovine serum (FBS), and 100 IU/mL penicillin G, 100 $\mu$g/mL streptomycin, and 0.25 mg/mL amphotericin B. The medium was then replaced and cells were treated for 48 hours with $T_3$ (100 nM) using PRL (1 $\mu$g/mL), $PGE_2$ (1 $\mu$M), and luteinizing hormone (LH; 100 ng/mL) as stimulators. Each experimental condition was performed in triplicate and the experiment was repeated twice. The concentrations used for $T_3$, PRL, $PGE_2$, and LH were chosen based on previous published data (28–30). At the end of the experiment, cells were centrifuged, and the culture medium was frozen at −20°C for subsequent RIA of $P_4$.

For primary luteal cell culture, we used the method described by Nelson *et al.* (29). Briefly, on day 19 of pregnancy, three control or hypothyroid rats were killed by decapitation, ovaries were removed, and CL dissected. The CLs were placed in Hanks balanced salt solution (HBSS) media containing 2% bovine serum albumin (BSA) and 25 mM HEPES, pH 7.4. Any follicle adhering to the CL was carefully removed. CL were incubated at 37°C with 50 U/mL collagenase, 2.4 U/mL dispase, and 200 U/mL DNase in four consecutive incubations (30 minutes each), with stirring at 100 rpm under an atmosphere of 100% $O_2$. After incubation, the cells were centrifuged at 200*g*, the supernatant was discarded, and fresh medium and enzymes were added. At the end of enzyme treatment, CL were treated for 15 minutes in 10 mL EDTA solution (0.02% EDTA w:v in phosphate buffered saline [PBS]) containing 2% BSA and 25 mM HEPES, pH 7.4. After this treatment, the cells were centrifuged at 200*g*. The new pellet was resuspended in 10 mL of dissection medium and filtered through nylon mesh. The viability was between 80% and 90% as determined by trypan blue staining. For all experiments, luteal cells were cultured at 37°C under 95% air: 5% $CO_2$ atmosphere, using $10^5$ viable cells per milliliter of culture medium (McCoy's 5A:Ham's F-12, 1:1). The cells were incubated for 6 hours with $T_3$ (1–500 nM) using pregnenolone (100 nM) as precursor for $P_4$ synthesis. At the end of each experiment, the cells were centrifuged, and the culture medium was frozen at −20°C for subsequent RIA of $P_4$.

### *Statistical analysis*

Statistical analysis was performed using one-way analysis of variance followed by Bonferroni *post hoc* test to compare between groups. When variances were not homogeneous, we performed log transformation of the data. In the cases in which the data do not present a Gaussian distribution after log transformation, the non-parametric Kruskal Wallis test, followed by Dunn *post hoc* test, was used to compare the groups. Differences between means were considered significant at the $p < 0.05$ level (31).

## Results

### *Hormonal profile of $T_3$, $T_4$, TSH, and $P_4$ levels during gestation and postpartum*

The present results (Fig. 1) confirm (12) that the thyroid-pituitary axis is regulated during gestation and postpartum in the rat. $T_4$ decreased significantly throughout gestation, and returned after parturition to levels similar to those found in early pregnancy (G5). Conversely, the levels of $T_3$ increased slightly on G15 and decreased thereafter, remaining at levels similar to those of early pregnancy (G5). TSH surged on G15 and decreased thereafter, but it remained at higher levels than during the first half of gestation. $P_4$ concentration was elevated throughout gestation and decreased on G21 due to the triggering of luteolysis concomitant with the onset of parturition. $P_4$ remained at low levels in the early postpartum period.

### *TRs mRNA expression at luteal levels during gestation and postpartum*

In order to evaluate for the first time a possible direct action of THs on CL function during pregnancy, the expression of thyroid hormone receptors was assessed. Figure 2 shows that in the rat, the CL of pregnancy express *TR$\alpha$1*, *TR$\alpha$2,* and *TR$\beta$1* mRNA, however, the presence of *TR$\beta$2*

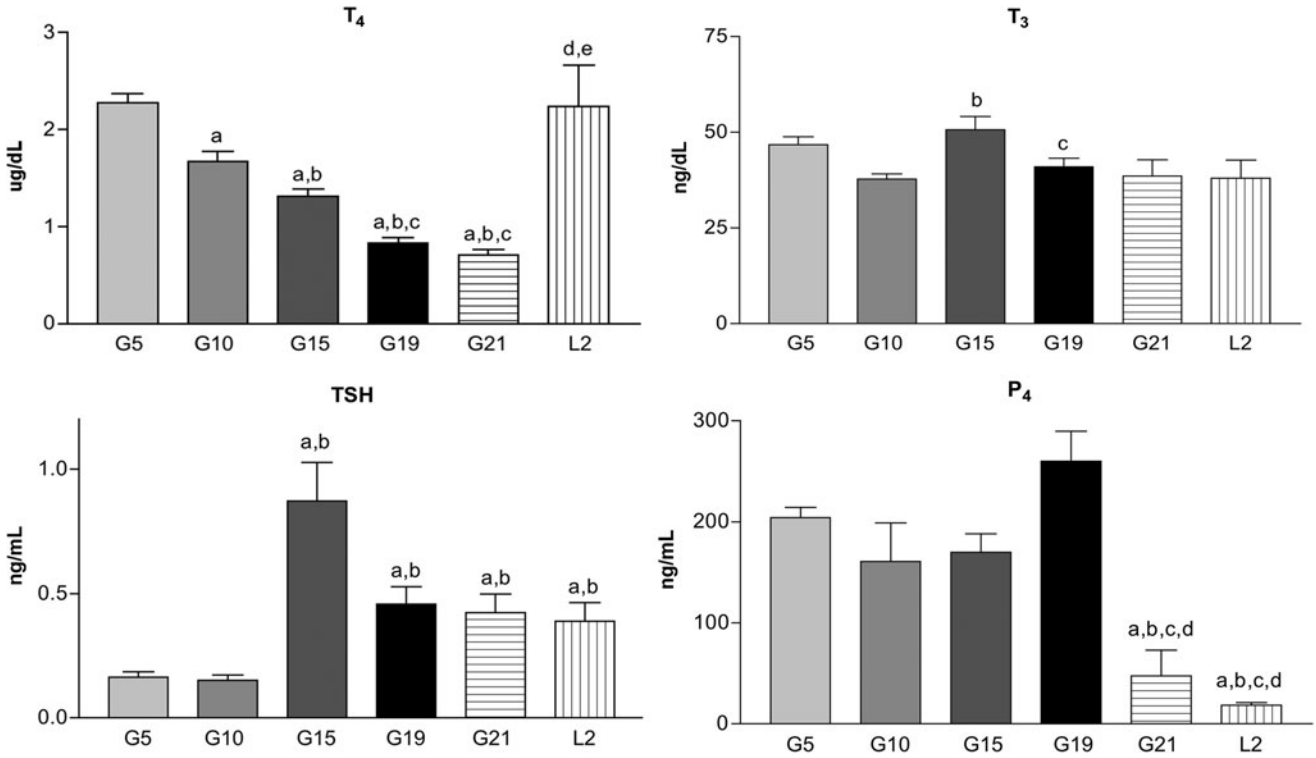


**FIG. 1.** $T_4$, $T_3$, TSH, and $P_4$ circulating concentrations during gestation and postpartum in the rat. Serum concentration of $T_4$, $T_3$, TSH, and $P_4$ assessed by RIA on G5 ($n=6$), G10 ($n=6$), G15 ($n=6$), G19 ($n=6$), G21 ($n=9$), and L2 ($n=6$). Results are shown as means ± SEM of groups of 6–9 rats. To compare $T_3$ and TSH after log transformation, one-way ANOVA and Bonferroni posttest was used to compare between groups. To compare $P_4$ and $T_4$, Kruskal Wallis and Dunn posttest was used to compare between groups. Statistical significance ($p<0.05$) is indicated: [a]vs. G5, [b]vs. G10, [c]vs. G15, [d]vs. G19, and [e]vs. G21. $T_4$, thyroxine; $T_3$, triiodothyronine; TSH, thyrotropin; $P_4$, progesterone; RIA, radioimmunoassay; G#, gestational day #; L2, day 2 of lactation; SEM, standard error of the mean; ANOVA, analysis of variance.

was not detectable (data not shown). *TRα1* mRNA expression increased during the first two-thirds of gestation reaching a peak at G15, and decreased significantly at G19, remaining at levels similar to those of G5 at the end of gestation and after parturition. *TRα2* mRNA expression was similar on all the days of pregnancy studied, with the exception of a peak observed on G10, although the differences were only significant compared with G19 and G21. *TRβ1* mRNA expression tended to increase in the first part of gestation; on G19 the expression decreased significantly compared with G10, and it increased gradually thereafter reaching significant differences in the postpartum period with respect to G19.

### *TRα1 and TRβ1 protein expression in CL during gestation and postpartum and its regulation by thyroid status in the rat*

The two most widely expressed and best characterized isoforms that bind $T_3$ are TRα1 and TRβ1. Because the normal expression of these proteins has never been identified in the CL, we decided to determine their luteal expression at protein level during gestation and postpartum. We identified the presence of TRβ1, which is abundantly expressed during gestation and postpartum in CL (Fig. 3). The expression of this receptor slightly increased during gestation reaching a peak on G19 and decreasing significantly on the last day of gestation and postpartum. On the contrary, TRα1 is barely expressed at the beginning of gestation and increased significantly with respect to the expression on G19 as well as compared to TRβ1; its expression diminished thereafter (Fig. 3).

Due to the increased expression of both TR isoforms found on G19 we explored the influence of thyroid status on their expression at luteal level on this day. To further corroborate the specificity of the signals, we used specific antibodies for TRβ1 (J-52) and TRα1 (PAI-211) that confirmed the results shown in Figure 3 and demonstrated that CL express TRβ1 and TRα1 (Fig. 4). To determine if thyroid status can modulate the expression of TRs at luteal level, as has been shown for other rat tissues such as myocardium and epididymis (22,32), we determined the expression of TRβ1 and TRα1 at the protein and mRNA level at G19 in control, hypothyroid and hyperthyroid rats. Our results show that thyroid status does not affect the expression of luteal TR at this stage of pregnancy since the expression of TRβ1 and TRα1 at the protein (Fig. 4) and mRNA level (Supplementary Fig. S1) remained unchanged. Hyperthyroid and hypothyroid status were confirmed measuring circulating $T_4$ and TSH. Hyperthyroid rats had significantly increased $T_4$ and decreased TSH, while hypothyroid rats had decreased $T_4$ and increased TSH serum concentrations compared to the control group (Supplementary Fig. S2).

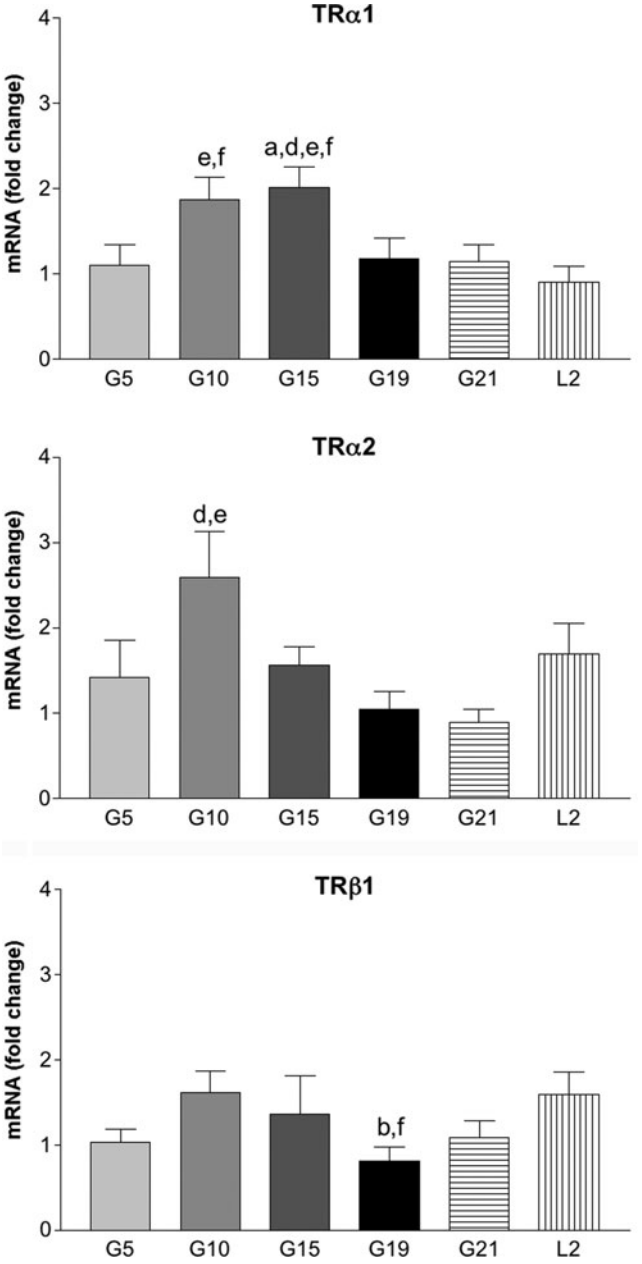


**FIG. 2.** TR$\alpha$1, TR$\alpha$2, and TR$\beta$1 luteal mRNA expression. mRNA fold change with respect to G5 and normalized to gapdh estimated by real-time RT-PCR during gestation and postpartum in the rat. The results are shown as means ± SEM of groups of 5 rats. One-way ANOVA and Bonferroni posttest were used to compare between groups to estimate TR$\beta$1 expression, and nonparametric Kruskal Wallis and Dunn posttest were used to compare between groups for TR$\alpha$1 and TR$\beta$2. Statistical significance ($p < 0.05$) is indicated: [a]vs. G5, [b]vs. G10, [c]vs. G15, [d]vs. G19, and [e]vs. G21. RT-PCR, reverse transcription-polymerase chain reaction.

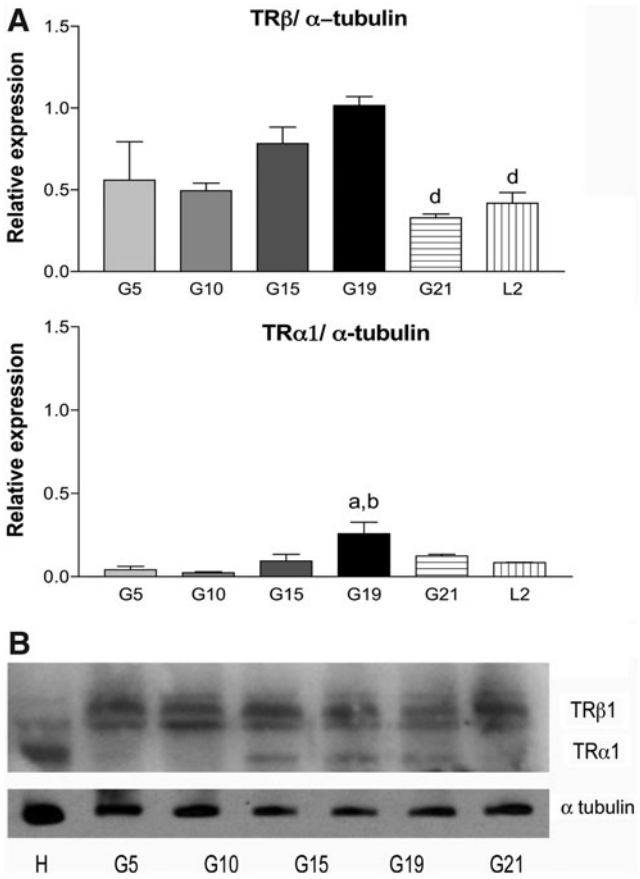


**FIG. 3.** Luteal TR protein expression throughout gestation in the rat. **(A)** Western blot of TR$\alpha$1 and TR$\beta$1 proteins on whole luteal protein extracts, immunoblotted against TR$\alpha$1 and TR$\beta$1 (48 and 55 kDa, respectively) and $\beta$-tubulin (55 kDa) antisera. Relative quantitation with respect to $\beta$-tubulin; results are expressed as the mean ± SEM of groups of three rats. Kruskal Wallis nonparametric test followed by Dunn posttest was used to compare between groups. Statistical significance ($p < 0.05$) is indicated: [a]vs. G5, [b]vs. G10, and [d]vs. G19. **(B)** Chemiluminescence image of a representative blot using Image Lab 4.0 software (Bio-Rad Laboratories).

### *Immunolocalization of TRs in CL of gestation in hypothyroid, hyperthyroid, and control rats*

In order to confirm the presence of the receptor and to evaluate its cellular localization we determined the presence of TR$\alpha$1 in the CL of gestation by IHC (Fig. 5). The immunostaining signal observed was moderate in CL and intense in granulosa cells of follicles within the ovary. The immunolocalization was predominantly nuclear as expected. When comparing the presence and localization of the mentioned isoform in CL of gestation in hypothyroid, hyperthyroid and control rats, we did not observe any significant difference due to thyroid status. Although we performed the experiments evaluating the immunolocalization of TR$\alpha$1 and $\alpha$2, the antibodies tested did not give a reliable signal and were not considered for this study.

### *Progesterone synthesis stimulation* in vitro

As a first step to understand the action of $T_3$ on CL function during pregnancy, we studied whether they are able to stimulate $P_4$ output *in vitro* using two different approaches. First, we assessed the capacity of the CL of pregnancy to produce $P_4$ in a primary culture in the presence of $T_3$, using as substrate pregnenolone (P5) and comparing the capacity of normal CL and those from hypothyroid rats (Fig. 6). The

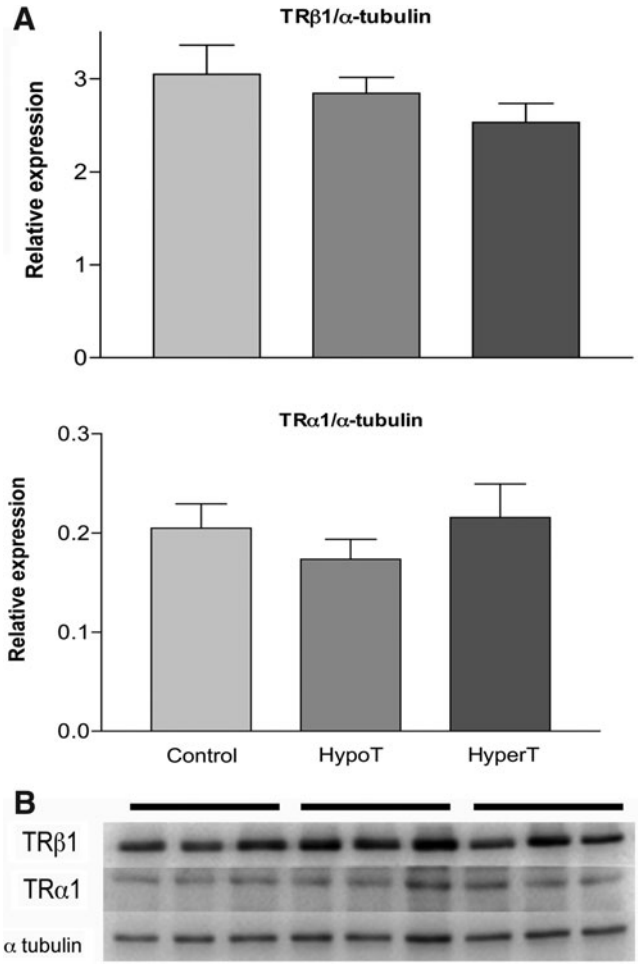


**FIG. 4.** Luteal TR protein expression at the end of pregnancy in euthyroid, hypothyroid, and hyperthyroid rats. **(A)** Western blot of TR$\alpha$1 and TR$\beta$1 proteins on whole luteal protein extracts, immunoblotted against TR$\alpha$1 and TR$\beta$1 (48 and 55 kDa, respectively) and $\beta$-tubulin (55 kDa) antisera. Results are expressed as the mean ± SEM of groups of three rats using one-way ANOVA and Bonferroni posttest to compare between groups. **(B)** Chemiluminiscence image of a representative blot using Image Lab 4.0 software (Bio-Rad Laboratories). HypoT, hypothyroid; hyperT, hyperthyroid.

experiment shows that $T_3$ alone did not modify the output of $P_4$ by the CL of pregnancy. Although, the percentage of $P_4$ output was always higher in the hypothyroid CL of pregnancy, the difference never achieved significance. Since CL of pregnancy cultures are difficult to maintain for long periods of time, we decided to attempt a second approach. We evaluated the action of $T_3$ on $P_4$ output using luteinized granulosa cell culture; these cells are more resistant in culture and therefore we could test their basal and stimulated capacity to synthesize $P_4$ for 48 hours, using different hormones that are established $P_4$ synthesis stimulators like PRL, LH and $PGE_2$. The analysis of $P_4$ in the culture media showed that $PGE_2$ and LH significantly increased the synthesis of $P_4$ compared to basal condition and under the stimulation with PRL. Although PRL stimulated $P_4$ synthesis, it did not achieve a significant difference compared to the controls. Using this model, we did not observe any significant effect of $T_3$ addition on basal nor stimulated $P_4$ synthesis (Fig. 6).

## Discussion

Experimental findings indicate that adequate circulating TH levels are needed to support CL formation and pregnancy (5,6,11). Previous studies from our laboratory demonstrate that hypothyroidism impairs CL function during gestation, delaying the onset of parturition in the rat (12,13). On the contrary, hyperthyroidism advances $P_4$ withdrawal and the onset of parturition (14,15) suggesting that TH participates in the maintenance and function of CL of gestation in the rat.

The physiologic variation in TH levels during gestation responds to the demands of the fetus. The thyroid status during gestation, in this study, confirms the results we have reported before (12). The concentration of $T_4$ diminished as gestation progressed in order to maintain adequate $T_3$ levels to assure a proper TH environment for both maternal and fetal tissues. This regulation may be possible since it has been demonstrated that uterine deiodinase D2 expression increases during gestation favoring the metabolism of $T_4$ to $T_3$ (33).

To test the possibility of a direct action of TH on the CL, we determined the presence of TR mRNA and protein. As expected, our results demonstrated that the CL of pregnancy express mRNA for *TR$\alpha$1*, *TR$\alpha$2,* and *TR$\beta$1* but not *TR$\beta$2*, as has been reported in other rat tissues such as placenta, mammary gland, liver, and kidney (34–36). The expression profiles of *TR$\alpha$1*, *TR$\alpha$2*, and *TR$\beta$1* mRNAs are similar to the results previously described in placenta at the end of gestation (34). Our results show the expression profile of TRs during the entire lifespan of the CL, since the beginning of gestation until the moment of functional and structural luteolysis, immediately before and after parturition respectively. The relative expression of *TR$\alpha$1*, *TR$\alpha$2*, and *TR$\beta$1* transcripts increased accompanying the growth in size of the CL during the first half of gestation, and decreased significantly thereafter. *TR$\beta$1* expression remains elevated after parturition, while *TR$\alpha$* and *TR$\alpha$2* decline during luteolysis along with the size of the CL (37). The increase observed in the relative expression of these receptors during early gestation suggests a physiologic and positive regulation, and consequently a possible function during this period. The expression of the *TR$\alpha$2* transcript followed a similar pattern as *TR$\alpha$1* confirming that both isoforms are generated by the same promoter and may be under the same type of regulation during gestation at luteal level, as has been demonstrated in other tissues (38). Despite that *TR$\alpha$* isoforms are expressed under the control of the same promoter their actions are dissimilar. The TR$\alpha$2 isoform does not bind $T_3$ but interacts with the same DNA response elements as TR$\alpha$1 and TR$\beta$1 (38). This type of interaction may allow TR$\alpha$2 to attenuate the effect of TR$\alpha$1 and TR$\beta$1 receptors bound to their ligands (39). Taking all together, this evidence shows that TR$\alpha$1 and TR$\beta$1 may function as promoters of CL development and survival during gestation and TR$\alpha$2 may participate as a regulator of this process.

To further understand the functions of the TRs, we also evaluated the protein expression and regulation of the predominant receptors that mediate TH actions, TR$\alpha$1 and TR$\beta$1, during gestation and postpartum. The upregulation of TR$\alpha$1 and TR$\beta$1 expression on G19 together with the

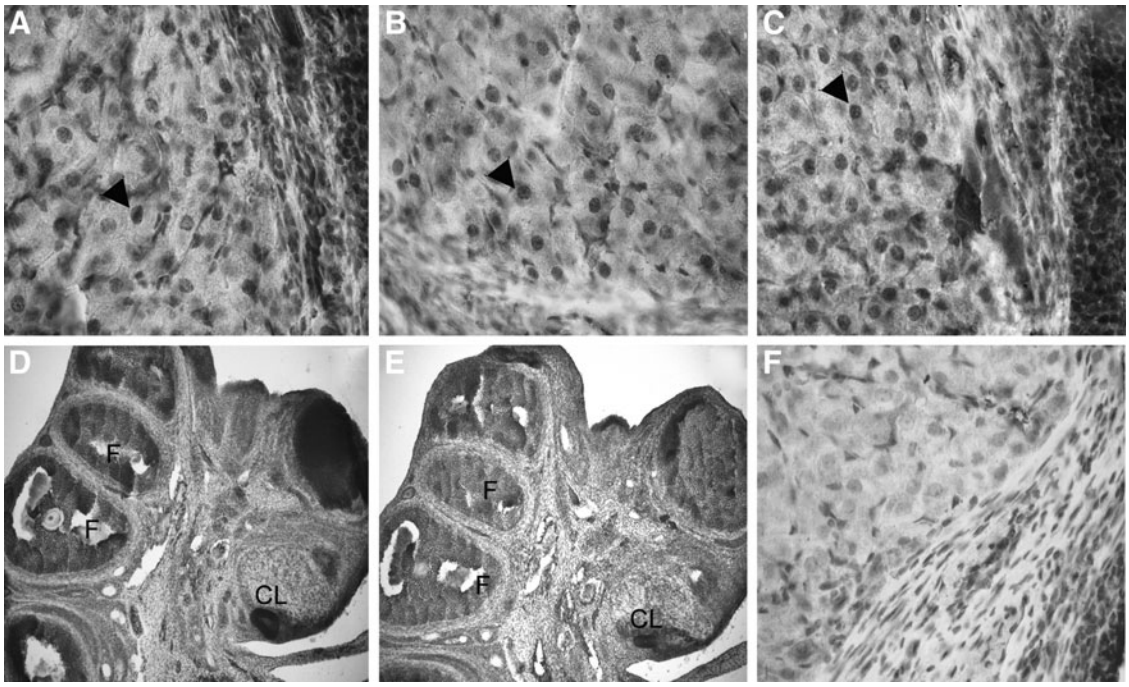


**FIG. 5.** Ovarian localization of TR$\beta$1 on day 19 of gestation in euthyroid, hypothyroid, and hyperthyroid rats. Immunostaining of a transversal section of the CL at 60× magnification in **(A)** control, **(B)** hypothyroid, and **(C)** hyperthyroid rats, respectively, arrows indicate the presence of nuclear staining in the CL of gestation. Transversal section of the ovary at 10× magnification in **(D)** treated with primary antisera and **(E)** negative control slide. **(F)** Negative control of CL without primary antisera at 60× magnification. CL, corpus luteum; F, follicle.

diminution of $T_4$, could respond to the necessity of maintaining adequate TH action in those tissues critical for the maintenance of pregnancy, among them, the CL function that drives the timing of parturition. The diminution of the luteal expression of TRs on day 21, a few hours before parturition, reinforces the hypothesis of a participation of TH on CL function and on the timing of parturition. Moreover, the presence of TR$\beta$1 on G19, as shown by immunohistochemistry, confirms its presence specifically in luteal cells and its nuclear localization, suggesting that such participation is mediated by a direct action as a transcription factor in this tissue.

The expression of TR$\alpha$1 and TR$\beta$1 protein throughout gestation showed a pattern similar to the profile of circulating $P_4$. Furthermore, on G21, when functional luteolysis has proceeded, evidenced by the prompt decline in $P_4$, the expression of TR$\alpha$1 and TR$\beta$1 descend concomitantly. The parallelism between $P_4$ and TRs expression allows us to speculate that $P_4$ may regulate the luteal expression of TR at the protein level but not at the posttranscriptional level, since the mRNA expression remains unchanged. At the end of gestation, when luteolysis is established, TR$\alpha$1 and TR$\beta$1 protein levels diminished significantly without significant changes in mRNA, suggesting that degradation of these proteins may be part of the several processes that take place during functional and structural luteolysis (37). On the other hand, luteal weight loss and the increase in number of apoptotic cells occur in the postpartum regressing CL, and are considered hallmarks of this process (40). $T_3$ has strong trophic actions in the ovary, since it promotes cell proliferation and survival in human granulosa cells, and prevents cell cycle arrest and induction of apoptosis in rat ovarian granulosa cells *in vitro* (41,42). These actions of TH on the ovary overlap with the prosurvival actions of $P_4$ on the ovary during gestation, which may be indicative of a possible interaction between both pathways. Thus, the fall in TR$\alpha$ and TR$\beta$ protein between G19 and G21 may be an early signal of luteolysis, and through the withdrawal of trophic signals, favor the subsequent regression and apoptosis of the luteal cells. Thus, TH action may not be further needed after functional luteolysis and consequently, the expression of TRs is downregulated.

The role of TH signaling on the reproductive process has been explored in other animal models. For example, knockin mice with the PV mutation in TR$\alpha$1, which abolishes $T_3$ binding and inhibits transactivation of the wild-type TRs, show dwarfism, increased mortality, reduced fertility, and mild thyroid failure (43). Reduced fertility was associated with decreased frequency of successful pregnancies and litter size, and this phenotype was attributed to the alteration in the receptor, since $T_3$ and $T_4$ showed concentrations slightly above normal values. Conversely, mice with a TR$\beta$1 with the PV mutation display a normal reproductive phenotype (44). Female mice lacking TR$\alpha$ and TR$\beta$ receptors rarely become pregnant and were deficient in nurturing any pups born (45). On the other hand, mice lacking either TR$\alpha$1 or TR$\beta$1 display normal fertility (46). The lack of concordance between the double knockout versus TR$\alpha$ or TR$\beta$ knockout suggests that both isoforms may compensate for each other and that they may have overlapping functions in the reproductive process. The discrepancies between the knockin and knockout models may be due because, at least *in vitro*, the mutation of the receptor in the knockin model exhibits a potent dominant

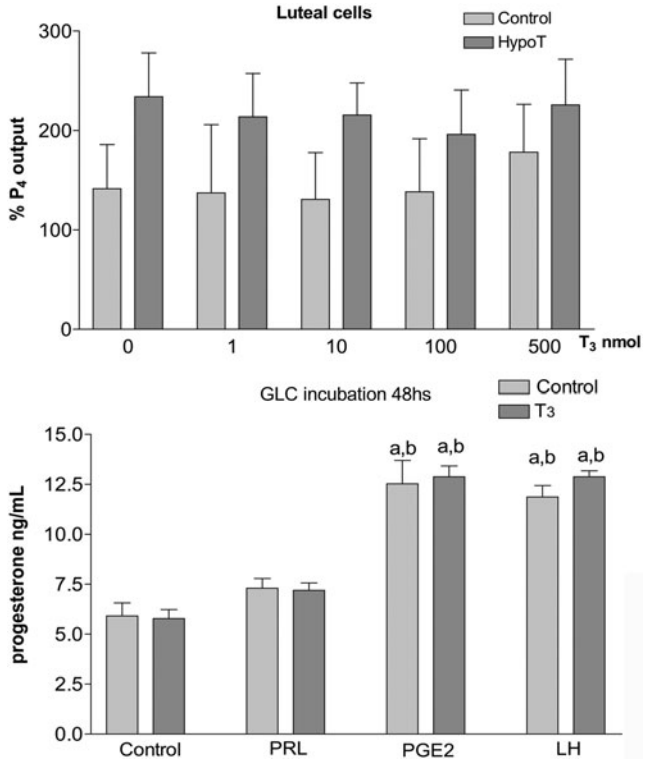


**FIG. 6.** *In vitro* effect of $T_3$ on luteal $P_4$ synthesis. **(A)** Production of $P_4$ by primary culture of CL on day 19 of gestation. Values represent the percentage as mean ± SEM of $P_4$ secreted to the media after 6 hours of $T_3$ treatment with respect to 0 hours using pregnenolone (100 nM) as precursor. Each point was performed in duplicates and the experiment was repeated three times. **(B)** Concentration of $P_4$ secreted to the culture media by granulosa luteal cells after 48 hours of $T_3$ treatment (100 nM) using PRL (1 $\mu$g/mL), $PGE_2$ (1 $\mu$M), and LH (100 ng/mL) as stimulators. Values are mean ± SEM, each point was performed in triplicate and the experiment was repeated twice. Statistical significance ($p < 0.05$) is indicated: [a]vs. control groups in the presence or absence of $T_3$; [b]with respect to the groups stimulated with PRL in the presence or absence of $T_3$ using one-way ANOVA and Bonferroni posttest to compare between groups. PRL, prolactin; $PGE_2$, prostaglandin $E_2$; LH, luteinizing hormone.

negative activity inhibiting the wild-type TRs transcriptional activity (44). Unfortunately, none of these studies identify which are the target organ/s affected by TH signaling that may be, at least in part, responsible for the alteration in the reproductive performance.

To our knowledge, this is the first study demonstrating expression and regulation of TR mRNA and protein in the CL of pregnancy in mammals. Despite TR presence and regulation during gestation on the CL, our *in vitro* results from whole luteal cell culture, failed to demonstrate a direct association between TH signaling and the main function of CL in the rat, namely, $P_4$ synthesis on day 19 of gestation. The lack of an association between TH signaling and $P_4$ synthesis may be due to the fact that the presence of blood and nonluteal cells in the culture may modify the responsiveness of luteal cells to $P_4$-inducing factors. It is tempting to speculate that TH may exert another physiological function in CL such as the regulation of cell cycle or survival. Furthermore, some effects may be indirect through alterations in prostaglandin synthesis and metabolism in other target tissues, which, in turn, affect functional luteolysis, as we have previously demonstrated (13,14).

In conclusion, in this study we describe the expression of TR isoforms in the CL during pregnancy and the postpartum period, identifying the CL of pregnancy as a TH target organ during gestation. TRs protein expression is modulated in this tissue in coincidence with the regulation of $P_4$ metabolism, and the abrupt changes before and after delivery suggest a role for THs during luteolysis. However, TH actions on the CL do not seem to be related to a direct regulation of $P_4$ synthesis. Due to the consequences of thyroid dysfunction on domestic animal and human reproduction, the challenge remains to elucidate the exact functions of TH on CL and their implication on reproductive performance.

## Acknowledgments

This work has been supported by grants PIP 2298/09 from CONICET (Consejo Nacional de Investigaciones Científicas y Técnicas, Argentina), PICT-R 32529 from the Agencia de Promoción Científica y Tecnológica, and SeCTyP 06/ M045. Universidad Nacional de Cuyo. Argentina. DFC-C, LMV-R, SRV, GAJ and MBH are Career Scientists from CONICET, PBN and ALR have fellowships from CONICET. The authors thank Dr. Parlow and the NHPP (National Hormone and Pituitary Program, Harbor–UCLA Medical Center, Torrance, California) for the materials they provided for the double-antibody RIA to measure TSH. The authors are grateful to Tiffany Weidner for the editing of the manuscript and Norma Carreño for her invaluable technical assistance.

## Author Disclosure Statement

No competing financial interests exist.

## References

1. Stocco C, Tellería CM, Gibori G 2007 The molecular control of corpus luteum formation, function, and regression. Endocr Rev **28:**32.
2. Davis JS, Rueda BR 2002 The corpus luteum: an ovarian structure with maternal instincts and suicidal tendencies. Front Biosci **7:**d1949–1978.
3. Arredondo F, Noble LS 2006 Endocrinology of recurrent pregnancy loss. Semin Reprod Med **24:**33–39.
4. McNeely MJ, Soules MR 1988 The diagnosis of luteal phase deficiency: a critical review. Fertil Steril **50:**1–15.
5. Maruo T, Hayashi M, Matsuo H, Yamamoto T, Okada H, Mochizuki M 1987 The role of thyroid hormone as a biological amplifier of the actions of follicle-stimulating hormone in the functional differentiation of cultured porcine granulosa cells. Endocrinology **121:**1233–1241.
6. Jiang JY, Imai Y, Umezu M, Sato E 2001 Characteristics of infertility in female hypothyroid (hyt) mice. Reproduction **122:**695–700.
7. Anderson LL, Hard DL, Carpenter LS, Awotwi EK, Diekman MA, Trenkle AH, Cho J 1999 Pregnancy, parturition, and lactation in hypophyseal stalk-transected beef heifers. J Endocrinol **163:**463–475.
8. LaFranchi SH, Haddow JE, Hollowell JG 2005 Is thyroid inadequacy during gestation a risk factor for adverse pregnancy and developmental outcomes? Thyroid **15:**60–71.

9. Bernal A, DeMoraes GV, Thrift TA, Willard CC, Randel RD 1999 Effects of induced hypothyroidism on ovarian response to superovulation in Brahman (Bos indicus) cows. J Anim Sci **77:**2749–2756.
10. Krassas GE, Poppe K, Glinoer D 2010 Thyroid function and human reproductive health. Endocr Rev **31:**702–755.
11. Naficy H, Behjatnia Y 1975 The effect of thyroid extract on luteal phase deficiency. Acta Med Iran **18:**55–60.
12. Hapon MB, Simoncini M, Via G, Jahn GA 2003 Effect of hypothyroidism on hormone profiles in virgin, pregnant and lactating rats, and on lactation. Reproduction **126:**371–382.
13. Hapon MB, Motta AB, Ezquer M, Bonafede M, Jahn GA 2007 Hypothyroidism prolongs corpus luteum function in the pregnant rat. Reproduction **133:**197–205.
14. Navas PB, Motta AB, Hapon MB, Jahn GA 2011 Hyperthyroidism advances luteolysis in the pregnant rat through changes in prostaglandin balance. Fertil Steril **96:**1008–1014.
15. Rosato RR, Gimenez MS, Jahn GA 1992 Effects of chronic thyroid hormone administration on pregnancy, lactogenesis and lactation in the rat. Acta Endocrinol (Copenh) **127:** 547–554.
16. Rae MT, Gubbay O, Kostogiannou A, Price D, Critchley HO, Hillier SG 2007 Thyroid hormone signaling in human ovarian surface epithelial cells. J Clin Endocrinol Metab **92:**322–327.
17. Zhang SS, Carrillo AJ, Darling DS 1997 Expression of multiple thyroid hormone receptor mRNAs in human oocytes, cumulus cells, and granulosa cells. Mol Hum Reprod **3:**555–562.
18. Wakim AN, Polizotto SL, Burholt DR 1994 Alpha-1 and beta-1 thyroid hormone receptors on human granulosa cells. Recent Prog Horm Res **49:**377–381.
19. Wakim AN, Polizotto SL, Buffo MJ, Marrero MA, Burholt DR 1993 Thyroid hormones in human follicular fluid and thyroid hormone receptors in human granulosa cells. Fertil Steril **59:**1187–1190.
20. Bandyopadhyay A, Roy P, Bhattacharya S 1996 Thyroid hormone induces the synthesis of a putative protein in the rat granulosa cell which stimulates progesterone release. J Endocrinol **150:**309–318.
21. Datta M, Roy P, Banerjee J, Bhattacharya S 1998 Thyroid hormone stimulates progesterone release from human luteal cells by generating a proteinaceous factor. J Endocrinol **158:**319–325.
22. Liu CR, Li LY, Shi F, Zang XY, Liu YM, Sun Y, Kan BH 2007 Effects of hyper- and hypothyroid on expression of thyroid hormone receptor mRNA in rat myocardium. J Endocrinol **195:**429–438.
23. Lardizabal MN, Nocito AL, Daniele SM, Ornella LA, Palatnik JF, Veggi LM 2012 Reference genes for real-time PCR quantification of microRNAs and messenger RNAs in rat models of hepatotoxicity. PLoS One **7:**e36323.
24. Varas SM, Munoz EM, Hapon MB, Aguilera Merlo CI, Gimenez MS, Jahn GA 2002 Hyperthyroidism and production of precocious involution in the mammary glands of lactating rats. Reproduction **124:**691–702.
25. Xing W, Deng M, Zhang J, Huang H, Dirsch O, Dahmen U 2009 Quantitative evaluation and selection of reference genes in a rat model of extended liver resection. J Biomol Tech **20:**109–115.
26. Livak KJ, Schmittgen TD 2001 Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method. Methods **25:**402–408.
27. Telleria CM, Goyeneche AA, Stocco CO, Gibori G 2004 Involvement of nuclear factor kappa B in the regulation of rat luteal function: potential roles as survival factor and inhibitor of 20alpha-hydroxysteroid dehydrogenase. J Mol Endocrinol **32:**365–383.
28. Horvath E, Varga B, Stark E 1986 Stimulation of progesterone production by adrenocorticotropic hormone and prostaglandin E2 in rat luteal cells. Biol Reprod **35:**44–48.
29. Nelson SE, McLean MP, Jayatilak PG, Gibori G 1992 Isolation, characterization, and culture of cell subpopulations forming the pregnant rat corpus luteum. Endocrinology **130:**954–966.
30. Cecconi S, Rucci N, Scaldaferri ML, Masciulli MP, Rossi G, Moretti C, D'Armiento M, Ulisse S 1999 Thyroid hormone effects on mouse oocyte maturation and granulosa cell aromatase activity. Endocrinology **140:**1783–1788.
31. Snedecor GWC, William Gemmell 1967 Statistical methods 6th ed. ed. Ames, Iowa State University Press
32. De Paul AL, Mukdsi JH, Pellizas CG, Montesinos M, Gutierrez S, Susperreguy S, Del Rio A, Maldonado CA, Torres AI 2008 Thyroid hormone receptor alpha 1-beta 1 expression in epididymal epithelium from euthyroid and hypothyroid rats. Histochem Cell Biol **129:**631–642.
33. Galton VA, Martinez E, Hernandez A, St Germain EA, Bates JM, St Germain DL 2001 The type 2 iodothyronine deiodinase is expressed in the rat uterus and induced during pregnancy. Endocrinology **142:**2123–2128.
34. Leonard AJ, Evans IM, Pickard MR, Bandopadhyay R, Sinha AK, Ekins RP 2001 Thyroid hormone receptor expression in rat placenta. Placenta **22:**353–359.
35. Macejova D, Baranova M, Liska J, Brtko J 2005 Expression of nuclear hormone receptors, their coregulators and type I iodothyronine 5'-deiodinase gene in mammary tissue of nonlactating and postlactating rats. Life Sci **77:**2584–2593.
36. Keijzer R, Blommaart PJ, Labruyere WT, Vermeulen JL, Doulabi BZ, Bakker O, Tibboel D, Lamers WH 2007 Expression of thyroid hormone receptors A and B in developing rat tissues; evidence for extensive posttranscriptional regulation. J Mol Endocrinol **38:**523–535.
37. Gonzalez-Fernandez R, Martinez-Galisteo E, Gaytan F, Barcena JA, Sanchez-Criado JE 2008 Changes in the proteome of functional and regressing corpus luteum during pregnancy and lactation in the rat. Biol Reprod **79:** 100–114.
38. Macchia PE, Takeuchi Y, Kawai T, Cua K, Gauthier K, Chassande O, Seo H, Hayashi Y, Samarut J, Murata Y, Weiss RE, Refetoff S 2001 Increased sensitivity to thyroid hormone in mice with complete deficiency of thyroid hormone receptor alpha. Proc Natl Acad Sci USA **98:**349–354.
39. Chassande O, Fraichard A, Gauthier K, Flamant F, Legrand C, Savatier P, Laudet V, Samarut J 1997 Identification of transcripts initiated from an internal promoter in the c-erbA alpha locus that encode inhibitors of retinoic acid receptor-alpha and triiodothyronine receptor activities. Mol Endocrinol **11:**1278–1290.
40. Goyeneche AA, Calvo V, Gibori G, Telleria CM 2002 Androstenedione interferes in luteal regression by inhibiting apoptosis and stimulating progesterone production. Biol Reprod **66:**1540–1547.
41. Verga Falzacappa C, Mangialardo C, Patriarca V, Bucci B, Amendola D, Raffa S, Torrisi MR, Silvestrini G, Ballanti P, Moriggi G, Stigliano A, Brunetti E, Toscano V, Misiti S

2009 Thyroid hormones induce cell proliferation and survival in ovarian granulosa cells COV434. J Cell Physiol **221:**242–253.
42. Verga Falzacappa C, Timperi E, Bucci B, Amendola D, Piergrossi P, D'Amico D, Santaguida MG, Centanni M, Misiti S 2012 T(3) preserves ovarian granulosa cells from chemotherapy-induced apoptosis. J Endocrinol **215:**281–289.
43. Kaneshige M, Suzuki H, Kaneshige K, Cheng J, Wimbrow H, Barlow C, Willingham MC, Cheng S 2001 A targeted dominant negative mutation of the thyroid hormone alpha 1 receptor causes increased mortality, infertility, and dwarfism in mice. Proc Natl Acad Sci USA **98:**15095–15100.
44. Cheng SY 2005 Isoform-dependent actions of thyroid hormone nuclear receptors: lessons from knockin mutant mice. Steroids **70:**450–454.
45. Gothe S, Wang Z, Ng L, Kindblom JM, Barros AC, Ohlsson C, Vennstrom B, Forrest D 1999 Mice devoid of all known thyroid hormone receptors are viable but exhibit disorders of the pituitary-thyroid axis, growth, and bone maturation. Genes Dev **13:**1329–1341.
46. Wikstrom L, Johansson C, Salto C, Barlow C, Campos Barros A, Baas F, Forrest D, Thoren P, Vennstrom B 1998 Abnormal heart rate and body temperature in mice lacking thyroid hormone receptor alpha 1. EMBO J **17:**455–461.

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